EFFECT OF ANTIOXIDANT TREATMENT ON CARDIAC HYPERTROPHY AND ANTIOXIDANT ENZYME EXPRESSION IN CARDIAC SPECIFIC GLUT4 DELETED MICE

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

We examined mice with cardiac specific deletion of the gene encoding glucose transporter 4 (GLUT4, $G4H^{-/-}$). These mice develop moderate cardiac hypertrophy with normal basal cardiac function and show signs of mitochondrial and whole cell oxidative stress as evidenced by increased concentration of oxidized glutathione (GSSG). Preliminary research in our laboratory has shown that treatment with the antioxidant tempol (a whole cell antioxidant), but not MnTBAP (a mitochondrial targeted superoxide 2 mimetic), can attenuate cardiac hypertrophy. Furthermore, GSSG concentrations were not altered by tempol, but reduced by MnTBAP. Based on these preliminary data, we hypothesized that 1) glutathione peroxidase (Gpx) was elevated and glutathione reductase (Gsr) was lower in hearts of $G4H^{-/-}$ mice. Both Gpx and Gsr are important components of glutathione cycle. 2) MnTBAP treatment increases Gsr and lowers Gpx expression, but tempol did not affect either of them. 3) Tempol treatment can decrease GSK3β phosphorylation and increase Txn2 expression.

In contrast to our hypothesis, Gpx was not elevated in $G4H^{-/-}$ mice and Gsr levels were similar between $G4H^{-/-}$ and control. Treatment with either tempol or MnTBAP did

not affect Gpx protein levels. On the other hand, tempol reduced Gsr in $G4H^{-/-}$ mice vs. untreated $G4H^{-/-}$ and controls. GSK3β activity was similar between $G4H^{-/-}$ mice and controls and remained unchanged after tempol and MnTBAP. Txn2 expression was also similar between controls and $G4H^{-/-}$. Tempol treatment increased Txn2 mRNA expression in controls only, but MnTBAP did not affect Txn2 in any group. Additionally, after tempol treatment, mRNA level of Gpx was not elevated in $G4H^{-/-}$ mice but actually lower, whereas Gsr was unchanged.

To sum up, the reduction of hypertrophy after tempol treatment was not associated with changes in; a) GSK3β phosphorylation, b) enzymes used in the glutathione oxidation and reduction cycle, or c) Txn2 expression. Additionally, though preliminary studies found a reduction of GSSG after MnTBAP treatment, the present study found this was not accompanied by any change in Gpx or Gsr. Therefore, we conclude that the attenuation of cardiac hypertrophy is unrelated to the modulation of oxidative stress by these enzymes, or a change in GSK3β phosphorylation.

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I. INTRODUCTION

From the most recent report of American Heart Association some 500,000 people have hypertrophic cardiomyopathy, yet most are unaware of it. [1] Hypertrophy can be a normal response to cardiovascular conditioning associated with exercise or pregnancy. This type of hypertrophy is classified as 'physiological', [2] and is distinguishable by its reversibility. In contrast, pathological cardiac hypertrophy is caused by the ventricle adapting to disease-inducing stimuli, such as chronically increased volume load (preload) or increased pressure load (afterload). Although it is an adaptation to the stress, prolonged stress-induced hypertrophy can lead the fibrosis and cardiac dilation, which is generally associated with symptoms of heart failure. [3]

Some data suggests that cardiovascular disease (CVD) risk appears to associate with the duration of diabetes mellitus (DM), with the rapidly rising global incidence of DM, and CVD is the major cause of morbidity and mortality in patients with diabetes. [4] Insulin resistance typically precedes the onset of type 2 diabetes and is accompanied by other cardiovascular risk factors, including dyslipidemia, hypertension, and prothrombotic factors. [5, 6] which commonly precede the development of type 2 diabetes by many years; [7] of great importance, the risk factors contribute independently to CVD

risk. Moreover, epidemiological and clinical studies report that diabetes can lead to cardiac hypertrophy independent from coronary artery disease, ischemia, or hypertension, [8-10] for reasons that are still unclear.

One possible explanation for hypertrophy in the prediabetic insulin-resistant heart is oxidative stress. Reactive oxygen species (ROS), which are a commonly reported feature in the diabetic heart, [11-13] have emerged as key mediators in cardiac pathophysiology. [14-20] Initially, insulin resistance is compensated by hyperinsulinemia through which a normal glucose tolerance is preserved. Deterioration to impaired glucose tolerance occurs when insulin resistance increases further and/or the compensatory insulin secretion decreases. An increase in insulin, free fatty acid, and/or glucose levels can increase ROS production and oxidative stress, as well as activate stress-sensitive pathways. [21] Many studies support that ROS generated by NADPH oxidase is a vital link in signaling pathways that stimulate cardiac hypertrophy. [22-31]

To study the effects of cardiac specific insulin resistance, a mouse model with cardiac specific GLUT4-deletion ($G4H^{-/-}$) was developed by Abel et al. [32] Different from global GLUT4 suppression, $G4H^{-/-}$ mice have reduced glucose uptake without interference from pathology stemming from systemic insulin resistance. In addition, moderate cardiac hypertrophy, without fibrosis, develops in $G4H^{-/-}$ mice by 8 weeks of age in the absence of hyperinsulinemia, hyperglycemia, or changes in serum concentrations of fatty acid, lactate, amino acids, or ketones. [32] Although these mice

have normal basal cardiac function, [32] contractile function after ischemic challenge is impaired. [33]

Preliminary work from our laboratory showed oxidative stress in hearts of G4H$^{-/-}$ mice as evidence by increased whole cell and mitochondrial oxidized glutathione concentrations. Interestingly, tempol (a whole cell antioxidant) treatment prevented cardiac hypertrophy in G4H$^{-/-}$ mice, but did not improve the ratio of reduced glutathione to oxidized glutathione (GSH:GSSG) nor blunt the increased expression of cardiac p67^{phox}, which is a subunit of NADPH oxidase. In contrast, MnTBAP (mitochondrial targeted superoxide 2 mimetic) treatment reduced mitochondrial concentrations of oxidized glutathione, but did not reduce cardiac hypertrophy. Therefore, in the present study, glutathione peroxidase (Gpx) and glutathione reductase (Gsr), important enzymes involved in glutathione cycle, were selected to observe. Gpx can convert hydrogen peroxide to water through oxidizing glutathione, and Gsr reduces GSSG to GSH.

Our preliminary studies using tempol treatment suggest that it is possible that in addition to oxidative stress, other mechanisms, independent of oxidative stress, may be operative in regulating cardiac hypertrophy in G4H$^{-/-}$ mice. For example, it has been proposed that attenuation of diabetic cardiomyopathy by tempol treatment is possibly associated to modulation of Glycogen synthase kinase 3β (GSK3β) and thioredoxin (Txn). [4, 38] Studies have shown that inactivation of GSK3 induces hypertrophic growth of cardiac myocytes by releasing transcription factors such as GATA4 and β-catenin, and

also by releasing eIF2B. A number of studies suggest that GSK3α and GSK3β, but GSK3β in particular, plays a pivotal role in cardiac hypertrophy. [34, 35] It is also possible that Txn2, a mitochondrial redox protein, may have played a role in modulation of hypertrophy, not only because of its antioxidant property for maintaining the mitochondrial redox state and scavenging mitochondrial ROS, but also because of its signaling function in modulating Apoptosis signal-regulating kinase 1 and transcription factors NF-κB involved in cell growth/apoptosis and inflammation. Studies have shown both suppression and overexpression of thioredoxin2 induce hypertrophy. [36, 37]

Given our preliminary data, we conducted this follow-up study to test three hypotheses. 1) Gpx is elevated and Gsr is lower in G4H$^{-/-}$ mice. 2) MnTBAP treatment increases Gsr and lowers Gpx expression, but tempol did not affect either of them. 3) Tempol treatment can decrease GSK3β phosphorylation and increase Txn2 expression.

II. MATERIALS AND METHODS

Animals

Mice with cardiac specific deletion of GLUT4 were generated by crossing mice bearing the modified GLUT4 gene containing loxP sites with transgenic mice with cardiac-specific expression of the enzyme cre recombinase in the laboratory of Dr. Dale Abel, University of Utah. Mice with normal cardiac GLUT4 expression were used as control (CON). All animals were housed in E. E. Jones Medical Science Building, University of Utah, and kept on a 12 h light/dark cycle, with room temperature 24 ℃ and all experiments were conducted on random fed mice. Mixed cohorts of male and female mice were used for all studies since both develop cardiac hypertrophy as previously described. [32]

Protocols

All protocols were approved by the University of Utah Institutional Animal Care and Use Committee. Mice were given treatment at 4 weeks old, when they start to develop cardiac hypertrophy. These mice were separated into two experiments. Each experiment contained four groups. In tempol experiment, there were CON (n=8), $G4H^{-/-}$ (n=7), CON+T (n=9), and $G4H^{-/-}$+T (n=8). Mice received either drinking water or

drinking water supplemented with the antioxidant tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, 1 mM). Daily fluid consumption was monitored throughout the 4-week treatment period. In MnTBAP experiment, there were CON (n=3), $G4H^{-/-}$ (n=3), CON+MnTBAP (n=4), and $G4H^{-/-}$+MnTBAP (n=5). Mice were given 200 μL injections of either saline (0.9%) or MnTBAP [(Mn(III)tetrakis(4-Benzoic acid) porphyrin chloride, 10 mg MnTBAP in 10 ml saline, stored in 4℃] every 2 days during the 4-week treatment period. At the end of the treatment, mice were anaesthetized (7% Chloride Hydrate, 0.1 mL/gram body weight), body weights (BW) recorded and hearts were excised and weighed (HW) to determine hypertrophy (HW/BW), and then tibia and part of tail excised. Tibia length (TL) was measured to determine hypertrophy (HW/TL). Heart samples were snap-frozen in liquid nitrogen and stored at -80℃.

Sample Preparation

All heart samples were trisected, ensuring that each portion contained a similar contribution of left and right ventricles. One tissue piece was homogenized in RIPA (RadioImmunoPrecipitation Assay, Tris-HCL, 50 mM, pH 7.4; NP-40, 1%; Na-deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM; Na3VO4, 1:200; NaF, 1:200; RIPA, 1:200; Protease inhibitor, 1:200) buffer. Then Bradford assay were conducted to detect the protein concentration of each sample. Forty μL dyes and 40 μL 1:100 diluted sample were added into 120 μL ddH2O to achieve the final dilution of 1:500. Absorbance

was measured at 595 nm. At last, sample loading buffer was added into each sample to bring the final concentration to 2 μg / μl and stored in 4 ℃ for western blot.

Western Blot

Glycine–SDS-PAGE (10% separating gel and 4% stacking gel) was used to separate proteins. Then gels were transferred to polyvinyl difluoride (PVDF) membrane. After blocking with 5% nonfat milk, primary antibodies for detecting protein of Gpx4 (CAYMAN CHEMICAL, Denver, CO), Gsr (Santa Cruz Biotechnology, Santa Cruz, CA), GSK3β, and p-GSK3β (Cell Signaling Technology, Beverly, MA) were incubated overnight in 1:1000 diluted with 5% nonfat milk or 5% BSA at 4 ℃. Secondary antibodies conjugated to goat anti-rabbit and anti-biotin (Cell Signaling Technology, Beverly, MA) were incubated in 1:5000 dilutions at room temperature for 1 hour. In order to avoid false conclusions due to unequal protein loading, we used loading controls for all Western blots and normalized target proteins to the density of tubulin (SIGMA-ALDRICH, St. Louis, MO). Therefore the errors in protein assay or loading were controlled. Signals were visualized by enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA). Relative band densities of immunoblots were measured using Kodak GL1500 imaging system and values were normalized to the mean control values.

RNA Extraction and RT-PCR

The following genes were examined using Real Time polymerase chain reaction (RT-PCR): Gpx1, Gpx4, Gsr and Txn2. The sequences of primers (5' to 3') used for RT-PCR were: Gpx1 (forward primer: GACTGGTGGTGCTCGGTT; reverse primer: TCACCATTCACTTCGCACTT), Gpx4 (forward primer: GCAGGAGCCAGGAAG TAATCAAGA; reverse primer: GCATCGTCCCCATTTACACA), Gsr (forward primer: GTGGCACTTGCGTGAATGTTG; reverse primer: GCACTGCTGTGTTCCACATTA CCTT) and Txn2 (forward primer: GTGTGGGCTTCCCTCACCTCT; reverse primer: GGCTGGGCTGGGCATTACTGTTAGA). Total RNA were extracted from heart samples of control and both antioxidant-treated groups using TRIzol reagent (Invitrogen, Carlsbad, California) and TissueLyser. Then they were purified using the RNAeasy total RNA isolation kit (Qiagen, Valencia, California) and reverse transcribed. Equal amounts of cDNA were subjected to real-time PCR using SYBR Green as a probe as previously described. [39] All transcripts were expressed relative to cyclophilin (Cphn), and values were normalized to the mean control value.

Statistics

SPSS for Windows® Graduate Pack (Version 15.0, Chicago, IL) was used to analyze the data. Significant difference among groups was compared by one-way analysis of variance (ANOVA). Significance will be accepted at $p < 0.05$. When a significant

value was obtained, post hoc test was conducted using Tukey to determine where within the individual groups the differences were. All data were expressed as means ± SEM.

III. RESULTS

Index of Cardiac Hypertrophy

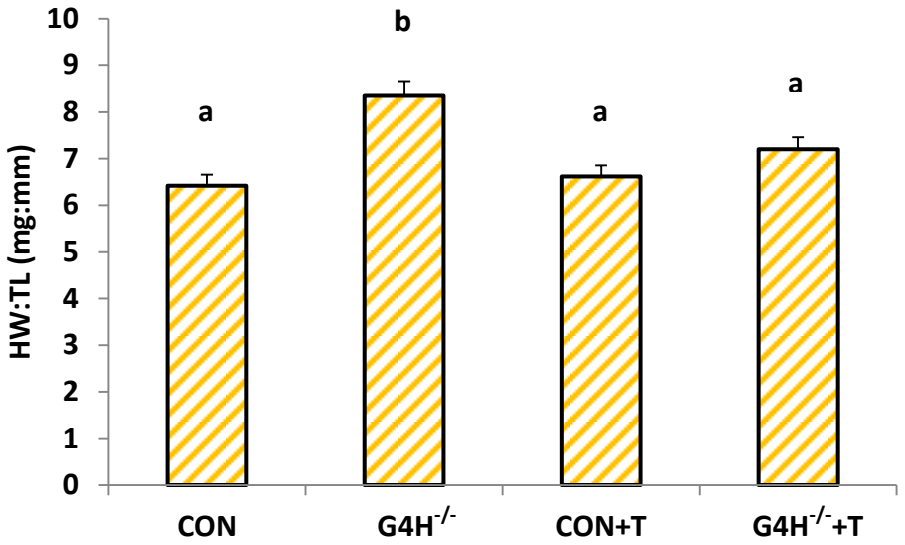
Heart weight was corrected to tibia length to create an index of cardiac hypertrophy. Tempol treatment, as compared with vehicle treated group, was associated with a significantly lower mean value of index of cardiac hypertrophy, as well as lower absolute heart weight (Table 1 and Figure 1). On the other hand, MnTBAP did not significantly change heart to tibia length ratios in $G4H^{-/-}$ mice, compared to the vehicle treated $G4H^{-/-}$ (Table 2 and Figure 2).

Table 1

Heart weight-to-body weight in tempol-treated and nontreated control and $G4H^{-/-}$ mice

	CON	**$G4H^{-/-}$**	**CON+T**	**$G4H^{-/-}$+T**
n	*n=15*	*n=13*	*n=14*	*n=13*
Heart Weight, mg	110.4±4.2[a]	143.9±5.7[b]	111.9±4.8[a]	121.1±4.8[a]
Body Weight, g	20.8±0.8	21.9±1.0	21.0±0.9	19.9±0.9
Tibia Length, mm	17.2±0.2	17.2±0.1	16.9±0.2	16.8±0.1

Values are means ± SE. Different letters denote significant difference, $p<0.05$.



Different letters denote significant difference, $p<0.05$.

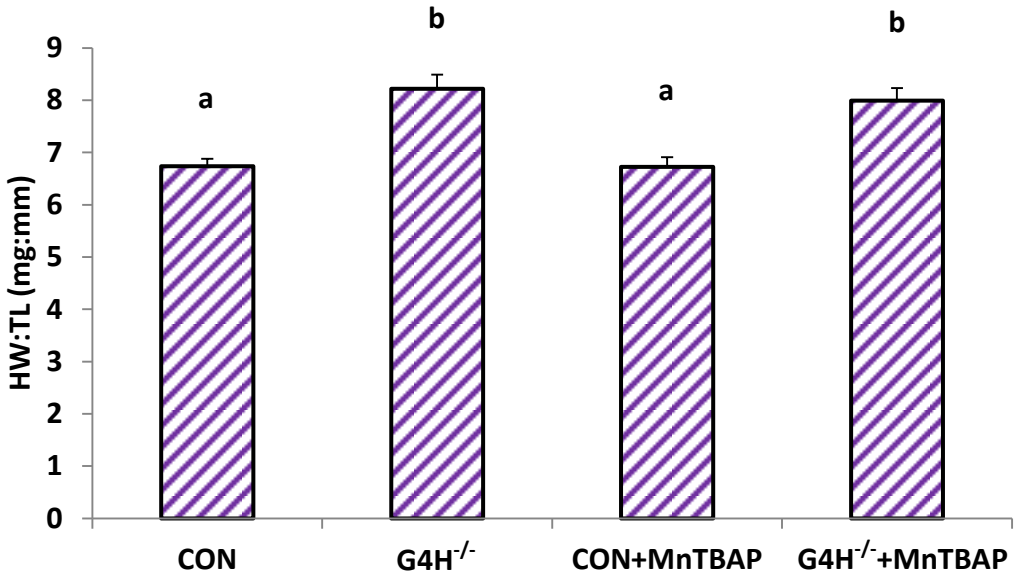
Figure 1. Heart weight-to-tibia length ratios in tempol-treated and nontreated control and $G4H^{-/-}$ mice

Table 2

Heart weight-to-body weight in MnTBAP-treated and nontreated control and $G4H^{-/-}$ mice

	CON	**$G4H^{-/-}$**	**CON+MnTBAP**	**$G4H^{-/-}$+MnTBAP**
n	*n=26*	*n=23*	*n=30*	*n=28*
Heart Weight, mg	118.0±3.3	131.0±11.4	120.0±7.9	128.4±10.9
Body Weight, g	24.2±2.8	21.3±1.3	23.0±1.8	20.4±2.2
n	*n=17*	*n=17*	*n=19*	*n=22*
Tibia Length, mm	16.5±0.2	16.6±0.1	16.6±0.1	16.4±0.1

Values are means ±SE.



Different letters denote significant difference, $p<0.05$.

Figure 2. Heart weight-to-tibia length in MnTBAP-treated and nontreated control and $G4H^{-/-}$ mice

Glutathione Peroxidase

Gpx is an antioxidant enzyme known to reduce phospholipid hydroperoxides. In this study, vehicle treated $G4H^{-/-}$ mice had greater mRNA, but lower protein level than controls (Figure 4 and Figure 5). However, this difference was only in $G4H^{-/-}$ vehicle treated mice used in the tempol study, since Gpx4 expression was similar in the $G4H^{-/-}$ vehicle treated cohort used in the MnTBAP study (Figure 7 and Figure 8). This difference may have been related to the smaller sample size used in MnTBAP studies. Tempol treatment did not change Gpx4 protein levels in control or $G4H^{-/-}$ mice vs. vehicle treated mice (Figure 4). However, Gpx4 mRNA level was lower in tempol treated

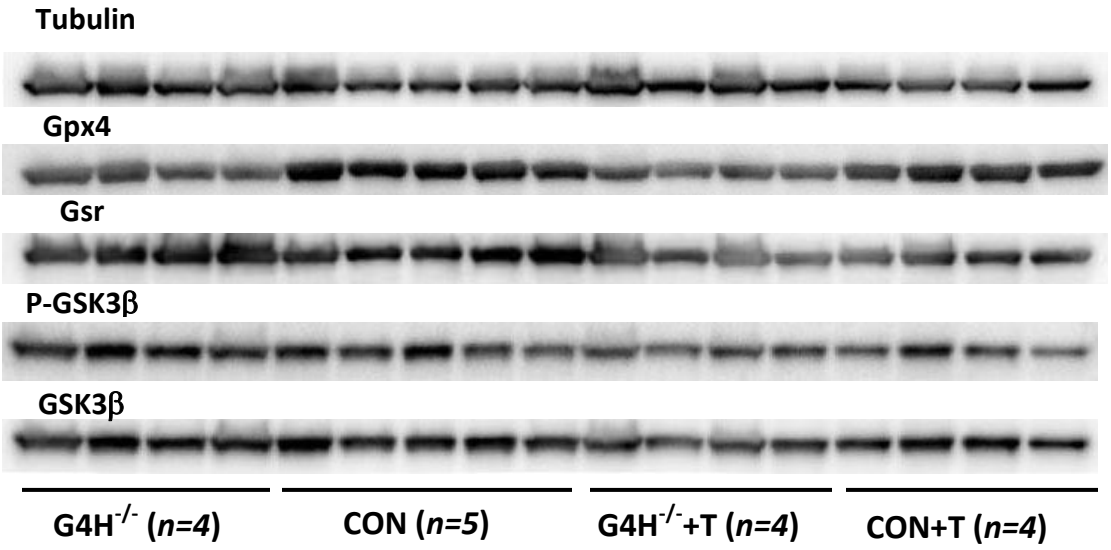
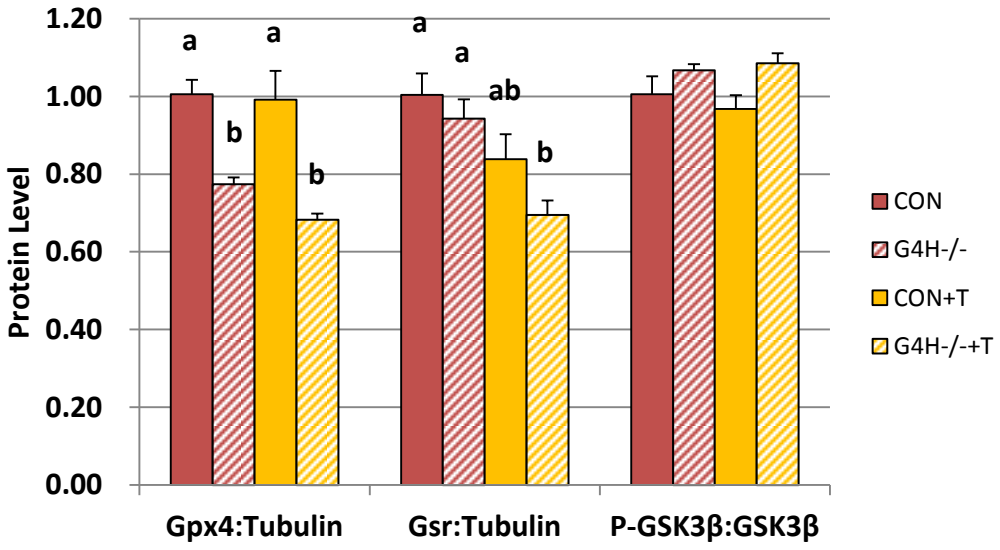
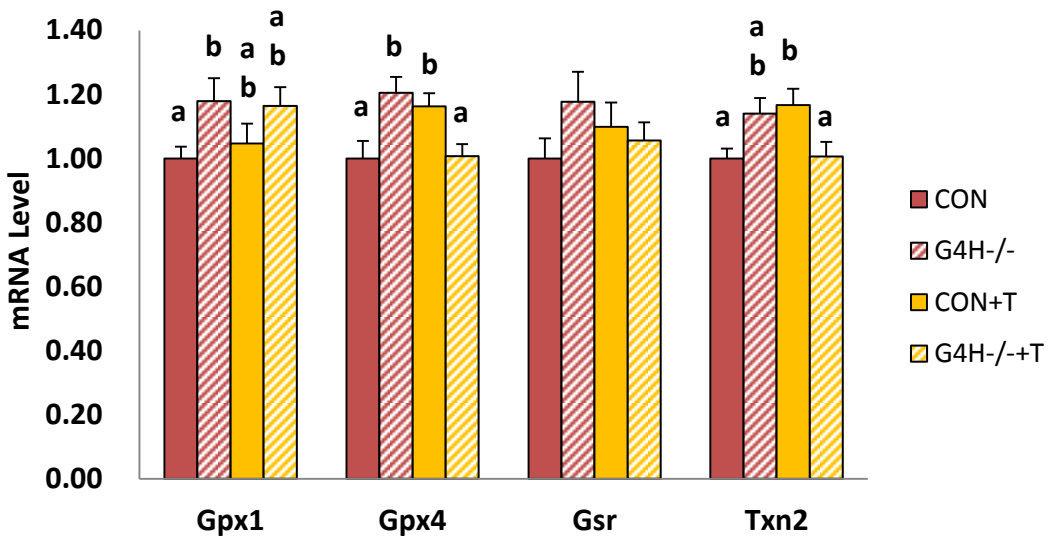


Figure 3. Western blots of Gpx4, Gsr and ratio of p-GSK3β and GSK3β in mice treated with and without tempol



All groups are normalized to the mean control value. Different letters denote significant difference, $p<0.05$.

Figure 4. mRNA expression of Gpx4, Gsr and Txn2 on both nontreated and tempol-treated mice



All groups are normalized to the mean control value. Different letters denote significant difference, $p<0.05$.

Figure 5. Quantification of Gpx4, Gsr and ratio of p-GSK3β and GSK3β on both nontreated and tempol-treated mice

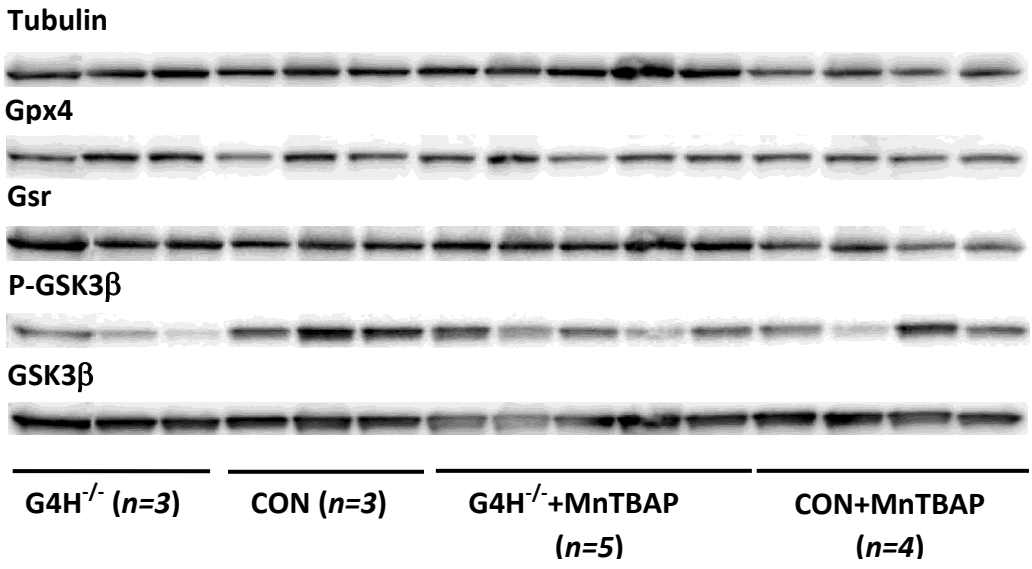
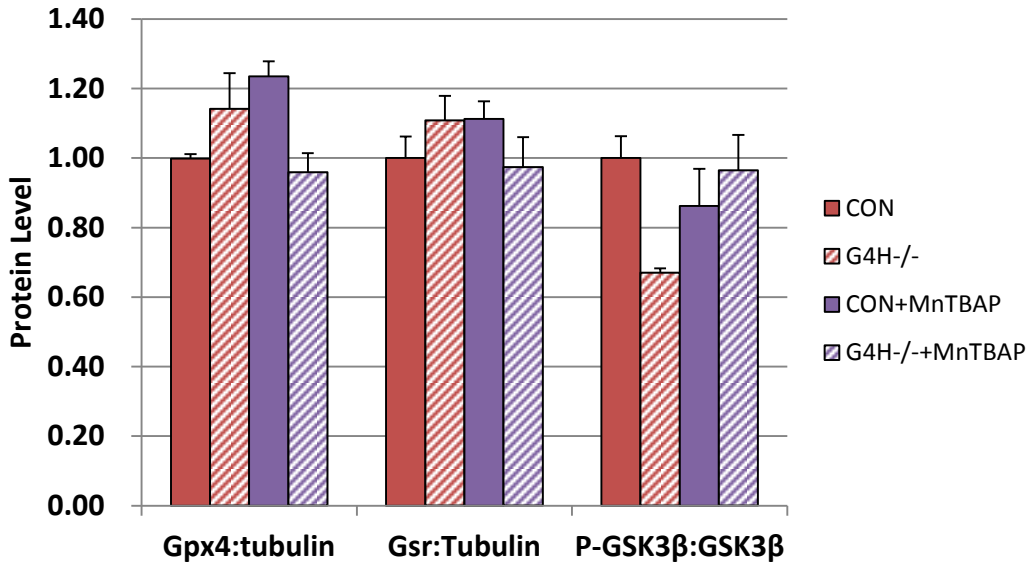
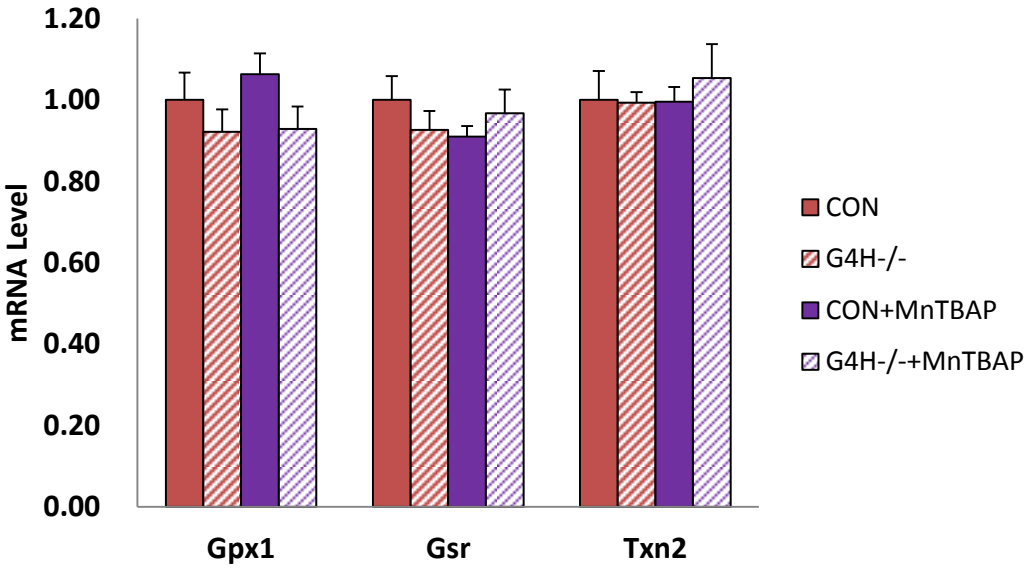


Figure 6. Western blots of Gpx4, Gsr and ratio of p-GSK and GSK in mice treated with and without MnTBAP



All groups are normalized to the mean control value.

Figure 7. mRNA expression of Gpx4, Gsr and Txn2 on both nontreated and MnTBAP-treated mice



All groups are normalized to mean control value.

Figure 8. Quantification of Gpx4, Gsr and ratio of p-GSK and GSK on both nontreated and MnTBAP-treated mice

$G4H^{-/-}$ mice compared to vehicle treated $G4H^{-/-}$ (Figure 5). MnTBAP treatment did not change Gpx protein or mRNA levels (Figure 7 and Figure 8).

Glutathione Reductase

Gsr is an important modulator of the glutathione oxidation-reduction cycle. Expression of Gsr mRNA and protein levels were similar between all untreated control and $G4H^{-/-}$ mice. Tempol treatment reduced Gsr protein levels in $G4H^{-/-}$ mice vs. vehicle treated control and $G4H^{-/-}$ mice (Figure 4). MnTBAP did not affect Gsr protein or mRNA level in control or $G4H^{-/-}$ mice (Figure 7 and 8).

Glycogen Synthase Kinase 3β Phosphorylation

Phosphorylation ratio of GSK3β was used to indicate GSK3β activity. Significant difference was neither observed in GSK3β phosphorylation between control and $G4H^{-/-}$ vehicle treated mice nor among tempol or MnTBAP treated mice (Figure 4 and 7). However, sample size for MnTBAP experiment was low and could not be increased due to unavailability of mice. Therefore, the statistical power might not strong enough to detect significance.

Thioredoxin 2

Txn2 protein was not detected by Western blot, perhaps due to a lack of antibody specificity in mouse heart. $G4H^{-/-}$ had similar Txn2 mRNA level, compared to control.

Tempol treatment increased the expression of Txn2 in control mice but did not alter it in $G4H^{-/-}$ mice (Figure 5). MnTBAP treatment did not result in any change in Txn2 mRNA level in control or $G4H^{-/-}$ mice (Figure 8).

IV. DISCUSSION

Cardiac Hypertrophy

In agreement with previous studies using $G4H^{-/-}$ mice, cardiac hypertrophy was observed in all untreated $G4H^{-/-}$ mice as evidenced by greater heart weight-to-tibia length than control mice. Tempol treatment significantly attenuated the hypertrophy in $G4H^{-/-}$ mice, while MnTBAP treatment did not. Our results are similar to a previous study done by Ritchie et al., who found that tempol treatment attenuated hypertrophy by decreasing heart body weight ratio, β-myosin heavy chain as well as B-type natriuretic peptide (BNP) in other models of cardiac specific GLUT4 deleted mice. [4]

Enzymes Used in the Glutathione Cycle

Preliminary work done in our laboratory found greater GSSG and lower GSH:GSSG in hearts of $G4H^{-/-}$ mice. Therefore in the present study Gpx and Gsr were measured at both the protein and mRNA level since both are involved in glutathione reduction and oxidation. While we hypothesized that $G4H^{-/-}$ mice would have higher levels of Gpx, our data was inconclusive since the untreated $G4H^{-/-}$ mice used in the tempol study had lower Gpx, while untreated $G4H^{-/-}$ mice used in the MnTBAP study

showed no change in Gpx. It is possible that the different sample size used in both studies may have led to these conflicting results. Regardless of whether Gpx was similar or lower in $G4H^{-/-}$ mice vs. controls, neither result supports our hypothesis that Gpx was greater in $G4H^{-/-}$ mice. Also in contrast to our hypothesis was the fact that MnTBAP treatment did not lead to any reduction in Gpx4 protein levels. Therefore it appears that the reduction of GSSG in MnTBAP treated $G4H^{-/-}$ mice observed in previous studies is not associated to a reduction in Gpx4. We speculate that elevated levels of other isoforms of Gpx, such as Gpx1, Gpx2, Gpx3 [40], as well as other enzyme, such as glutaredoxin [41], contributed to generate GSSG. To support this point, we did find that Gpx1 mRNA level was increased in the cohort of $G4H^{-/-}$ mice used in the tempol study. Unfortunately, we did not find different Gpx1 mRNA in another cohort, possibly due to the limited sample size used in the MnTBAP study. Also in contrast to our hypothesis, cardiac Gsr expression and protein levels were similar between control and $G4H^{-/-}$ mice. Furthermore, MnTBAP had no effect on either Gsr expression of control and $G4H^{-/-}$ mice. Taken together, our present data indicate that the reduction of GSSG after MnTBAP treatment in our previous study may result from an unknown mechanism, such as involvement of other Gpx or Gsr isoforms that were not measured in this study.

Tempol did not change Gpx in $G4H^{-/-}$ mice, which support the hypothesis. Although Gsr was reduced compared to vehicle treated $G4H^{-/-}$, similar protein levels of

this target were shown between control and G4H$^{-/-}$ after tempol treatment. This result is in line with similar GSSG concentration among tempol treated groups found in previous study. Therefore, it is likely that Gsr may not be a critical modulator of hypertrophy.

Compared to western blots, gene expression of Gpx and Gsr after tempol treatment demonstrated an inconsistent and more complicated pattern. We examined both Gpx1 and Gpx4. Gpx4 is the only major antioxidant enzyme known to directly reduce phospholipid hydroperoxides within membranes and lipoproteins. [42] Gpx1 is the most abundant, classical isoenzyme in the cytoplasm of nearly all mammalian tissues. In contrast to western blot assay to determine protein levels of Gpx4, we found that tempol raised Gpx4 in control mice and lowered it in G4H$^{-/-}$ mice. These results indicated that an unknown transcriptional event may be resulting in consistent amounts of protein being produced in spite of changes in mRNA levels.

Txn2 is a mitochondria-specific member of the thioredoxin superfamily. Mice with a cardiac-specific deletion of Txn2 by the loxP-Cre system developed a severe phenotype of hypertrophic cardiomyopathy [43] and overexpression of Txn2 attenuated ANG II induced hypertrophy. [36] Based on those studies, Txn2 was expected as an important antioxidant to fight against hypertrophy. However, in our study the mRNA level of Txn2 in untreated G4H$^{-/-}$ mice was similar to controls. A previous study by Ritchie et al. showed that mRNA level of Txn2 was upregulated in controls after

treatment with tempol [4], which is in line with our result. They also found that Tnx2 was increased in cardiac specific GLUT4 deleted mice after tempol treatment. [4] In contrast, we found that tempol did not affect Txn2 mRNA level in G4H$^{-/-}$ mice which indicates that attenuation of hypertrophy by tempol may be independent of Txn2. Our finding stands in contrast to previous studies that report Txn2 is tightly correlated to hypertrophy. However, since our study measured only Txn2 mRNA level, the true status of the actual levels of target Txn protein in the heart remains unknown.

Glycogen Synthase Kinase Pathway

GSK3β is known as a negative regulator of cardiac hypertrophy. Increased cellular activity of GSK3β produced by overexpression inhibits many aspects of cardiac hypertrophy, including increases in the rate of protein synthesis and hypertrophic gene expression. [44-48] Activation of the hypertrophic program by stimulation of β-adrenergic receptors, Gq-coupled receptors, and Fas receptors leads to inactivation of endogenous GSK3β via phosphorylation of Ser9, predominantly through the PI3K/Akt pathway. [45-49] We hypothesized that alterations in GSK may offer an alternative mechanism to explain the attenuation of hypertrophy following tempol treatment. However, our hypothesis was not supported since there was no difference in GSK3β phosphorylation between control and G4H$^{-/-}$ mice. Furthermore, treatment with either tempol or MnTBAP did not result in any further change in GSK3β. This result is in contrast to a study done by Ritchie et al. [4],

who reported that tempol treatment markedly upregulated expression of GSK3β mRNA in an alternative model of cardiac specific GLUT4 deleted mice. But both the Ritchie's et al. and our study did not found significant difference in GSK3β between control and $G4H^{-/-}$ mice. Therefore, the attenuation of cardiac hypertrophy in $G4H^{-/-}$ mice after tempol treatment may not result from modulation of GSK3β. However, protein change was not measured by Richtie et al., so it is unknown if the changes in mRNA actually led to relevant changes in protein.

The limitation of the present study is the small sample size used in the MnTBAP study. This may have contributed to the fact that data for Gpx were inconsistent among vehicle treated mice between the MnTBAP cohort and the tempol cohort. It also led to low statistic power to detect significance of GSK3β phosphorylation among vehicle treated mice. Another possible explanation for inconsistent Gpx data among these groups is the different vehicle treatment used between tempol and MnTBAP treatments. For example, compared with using drinking water as a vehicle during the tempol study, the vehicle saline injection used in the MnTBAP study may have caused some stress related effects that invalidate a comparison of vehicle treated controls between these two distinct cohorts. Other than the possibilities mentioned above, different parts of heart contained in the tissue pieces for western blot and quantification of mRNA probably led to inconsistent data between protein level and mRNA level of the targets.

In conclusion, the major findings of the present study are that a) the reduction of hypertrophy after tempol treatment is not related to changes in GSK3β activity or Txn2 expression, b) the reduction in GSSG concentrations observed in our previous studies after MnTBAP treatment is not associated to changes in glutathione oxidation and reduction cycle enzymes, Gpx and Gsr. Therefore, we speculate that the attenuation of cardiac hypertrophy in $G4H^{-/-}$ mice after tempol treatment may in fact result from mechanisms unrelated to oxidative stress. Further studies are needed to elucidate the mechanisms involved.

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